invitrogen™
by life technologies™

Expressway™ Lumio™ Cell-Free Expression and Detection System

For cell-free protein synthesis and fluorescence detection of recombinant proteins containing the Lumio™ tag

Catalog nos. K9900-60, V960-03, V960-04

Rev. Date: 27 September 2011
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MAN0000432

User Manual

Table of Contents

Kit Contents and Storage

Important

Kits have been reconfigured. New reagents are supplied and quantities of reagents are different. If you have used these products in the past, discard old versions of this manual and use the instructions provided in this manual.

Types of Kits

This manual is supplied with the following products.

Product	Amount*	Catalog no.
Expressway™ N-terminal Lumio™ Cell-Free Expression and Detection System	20 rxns	K9900-70
Expressway™ C-terminal Lumio™ Cell-Free Expression and Detection System	20 rxns	K9900-90
Expressway™ Lumio™ Cell-Free Expression and Detection System	20 rxns	K9900-60
pEXP3-DEST Vector Kit	40 µl	V960-03
pEXP4-DEST Vector Kit	40 µl	V960-04

* Amount based on 100 µl reaction size.

Kit Components

The Expressway™ Lumio™ Cell-Free Expression and Detection kits include the following components.

Component	Catalog no.				
	K9900-70	K9900-90	K9900-60	V960-03	V960-04
Expressway™ Mini Expression Module	√	√	√		
Expressway™ Mini Amino Acid Module	√	√	√		
Lumio™ Green Detection Kit	√	√	√		
BenchMark™ Fluorescent Protein Standard	√	√	√		
Control Vector (pEXP5-NT/CALML3)			√		
pEXP3-DEST Vector Kit	√			√	
pEXP4-DEST Vector Kit		√			√

For a detailed description of the contents of the Expressway™ Modules, see the next page. For additional information on the Lumio™ Green Detection Kit, refer to the manual supplied with the kit.

Kit Contents and Storage, continued

Shipping/Storage

The Expressway™ Lumio™ Cell-Free Expression and Detection System components are shipped on dry ice. Upon receipt, store as described below and on the next page.

Expressway™ Mini Expression Module

The following reagents are included in the Expressway™ Mini Expression Module. Note that the Expressway™ 2.5X IVPS Reaction Buffer (-A.A.) does not contain amino acids.

Store the entire box at -80°C or store individual components as listed below.

Item	Amount	Storage
E. coli slyD- Extract	400 µl	-80°C
2.5X IVPS Reaction Buffer (-A.A.)	400 µl	-80°C
2X IVPS Feed Buffer	500 µl	-80ºC
T7 Enzyme Mix	20 µl	-80°C -20°C after initial use
DNase/RNase-Free Distilled Water	1.75 ml	-20°C or -80°C

Expressway™ Mini Amino Acids Module

The following reagents are included in the Expressway™ Mini Amino Acids Module. **Store at -20°C.**

Item	Composition	Amount
Amino Acid Mix (-Methionine)	Contains all amino acids (50 mM) except for methionine in 50 mM HEPES, pH 11	160 µl
75 mM Methionine	75 mM in 50 mM HEPES, pH 7.5, 4 mM DTT	120 µl

Vectors

The Expressway™ Lumio™ vectors (included with Cat nos. K9900-70, K9900-90, V960-03, or V960-04) are listed below. **Store vectors at -20°C.**

Item	Composition	Amount
pEXP3-DEST	40 µL of 150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	6 µg
pEXP3-GW/CAT control	20 µL of 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	10 µg
pEXP4-DEST	40 µL of 150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	6 µg
pEXP4-ORF control	20 µL of 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	10 µg

Continued on next page

Kit Contents and Storage, continued

Lumio™ Green Detection Kit

The Lumio™ Green Detection Kit components are listed below.
Store reagents at -20°C.

Item	Composition	Amount
Lumio™ Green Detection Reagent	Proprietary	20 µl
Lumio™ Gel Sample Buffer (4X)	Proprietary	5 × 200 µl
Lumio™ In-Gel Detection Enhancer	Proprietary	200 µl

Expressway™ Control Vector

Catalog number K9900-60 comes with the pEXP5-NT/CALML3 control vector (10 µg) for use as a positive control for protein expression. The plasmid allows expression of an N-terminally-tagged calmodulin-like 3 (CALML3) fusion protein. The vector is supplied as 20 µl at 0.5 µg/µl in TE buffer, pH 8.0.
Store at -20°C.

Note: Catalog nos. K9900-70, K9900-90, V960-03, or V960-04 include an N- or C-terminal Lumio™ expression control plasmid.

BenchMark™ Fluorescent Protein Standard

BenchMark™ Fluorescent Protein Standard is supplied as seven distinct proteins (~11-155 kDa) in storage buffer (0.45 M Tris-HCl, pH 8.5; 2% SDS; 12% glycerol; 0.0025% Coomassie® G-250). Amount supplied is 125 µl.
To avoid freezing and thawing, aliquot in small volumes and store at -20°C, protected from light.

Additional Products

Accessory Products

Some of the reagents supplied in the Expressway™ Cell-Free *E. coli* Expression System as well as other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below. For more information, go to www.invitrogen.com or contact Technical Support (page 44).

Product	Quantity	Catalog no.
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions 20 reactions	C4040-10 C4040-03
Gateway® LR Clonase™ II Enzyme Mix	20 reactions	11791-020
DNase/RNase-Free Distilled Water	500 ml	10977-015
RNase *AWAY*™	250 ml	10328-011
Ampicillin	20 ml (10 mg/ml)	11593-019
Zeocin™	1 g	R250-01
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
PureLink™ PCR Purification Kit	50 reactions	K3100-01
Lumio™ Green Detection Kit	100 in-gel detections	LC6090
BenchMark™ Protein Ladder	2 x 250 µl	10747-012
BenchMark™ Fluorescent Protein Standard	125 µl	LC5928
SimplyBlue™ SafeStain	1 L	LC6060
One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells	10 reactions	A10460

Products to Detect Recombinant Fusion Protein

If you are expressing your Lumio™-tagged recombinant protein from pEXP3-DEST or pEXP4-DEST, you may detect expression of your recombinant fusion protein using the Lumio™ Detection Reagents supplied with the kit. You may also use an antibody to the appropriate epitope. The table below describes the products available from Invitrogen for detection of fusion proteins expressed from these vectors. The amount of antibody supplied is sufficient for 25 western blots.

Product	Epitope	Catalog no.
Anti-HisG Antibody	Detects the N-terminal polyhistidine (6xHis) tag followed by glycine: HHHHHHG	R940-25
Anti-HisG-HRP Antibody		R941-25
Anti-HisG-AP Antibody		R942-25

Additional Products, continued

Products to Purify Recombinant Fusion Protein

If you have expressed your protein of interest in frame with the N- or C-terminal polyhistidine (6xHis) tag, you may use a nickel-charged agarose resin such as ProBond™ or Ni-NTA to purify your recombinant fusion protein. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-chelating Resin	50 ml	R801-01
	150 ml	R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Agarose	10 ml	R901-01
	25 ml	R901-15
Purification Columns (10 ml polypropylene columns)	50	R640-50
AcTEV™ Protease (pEXP3-DEST only)	1,000 units	12575-015

Expressway™ Kits

Other Expressway™ Cell-Free *E. coli* Expression System Modules are available separately from Invitrogen. Ordering information is provided below. For more information, go to www.invitrogen.com or contact Technical Support (page 44).

Product	Quantity	Catalog no.
Expressway™ Mini Cell-free *E. coli* Expression System	1 kit	K9901-00
Expressway™ Maxi Cell-free *E. coli* Expression System	1 kit	K9900-97
Expressway™ Maxi Cell-free *E. coli* Expression System with pEXP5-TOPO® vectors	1 kit	K9900-96

Introduction

Overview

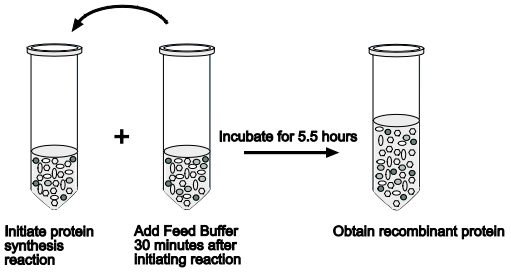
Introduction

The Expressway™ Lumio™ Cell-Free Expression and Detection System is designed for *in vitro* transcription and translation of target DNA to protein in a single tube, with the ability to perform both real-time and rapid in-gel detection of Lumio™-tagged recombinant proteins. Using this kit, your gene of interest is fused to the Lumio™ tag, enabling sensitive and specific in-gel detection of the Lumio™-tagged fusion protein in polyacrylamide gels without the need for staining or western blotting. You can also monitor real-time synthesis of the Lumio™-tagged fusion protein using a standard fluorometer.

The system uses an *E. coli* extract and a T7 Enzyme Mix that have been optimized for expressing full-length, active protein from DNA constructs in about 2 hours. Proteins with the Lumio™ tag can be detected during synthesis using real-time detection and after synthesis using in-gel detection.

How the System Works

The Expressway™ Cell-Free *E. coli* Expression System uses an optimized *E. coli* extract, a reaction buffer containing an ATP regenerating system, and amino acids to allow high-level synthesis of your recombinant protein of interest. At one or several time points after initiating the protein synthesis reaction, the reaction is supplemented with an optimized Feed Buffer containing a proprietary mixture of salts, amino acids, and other substrates that are depleted or degraded over time during protein synthesis (see Figure below). Addition of this Feed Buffer to the reaction replenishes these components and allows continuous cell-free protein synthesis to occur, resulting in the achievement of significantly enhanced recombinant protein yields in up to 2-6 hours.



Applications

The Expressway™ Lumio™ Cell-Free Expression and Detection System is suitable for use in the following applications:

Characterizing proteins

Analyzing mutants

Verifying cloned gene products

Producing proteins that are toxic to cells

Real-time detection of protein production

For more information on the downstream applications of cell free protein expression technologies, refer to published reviews (Katzen *et al.*, 2005).

Continued on next page

Overview, continued

Components of the System

An optimized S30 *E. coli* extract (Zubay, 1973) for increased stability of DNA constructs during transcription and translation and enhanced signal-to-background ratio with Lumio™ detection.

An optimized feed buffer containing salts and other substrates (Kim and Swartz, 1999) to replenish components depleted or degraded during protein synthesis, thus enhancing recombinant protein yield

Proprietary T7 Enzyme Mix containing T7 RNA polymerase and other components optimized for T7-based expression from DNA templates (Studier *et al.*, 1990)

Optimized reaction buffer composed of an ATP regenerating system to provide an energy source for protein synthesis (Kim *et al.*, 1996; Lesley *et al.*, 1991; Pratt, 1984)

Amino acids (- Met) required for protein synthesis to occur, and methionine provided separately for optimization of radiolabeling assays

Optimized expression vectors with an N-terminal (pEXP3-DEST) or C-terminal (pEXP4-DEST) Lumio™ tag for specific detection of fusion proteins using the Lumio™ Green Detection Reagent.

Features of pEXP3-DEST and pEXP4-DEST Vectors

The pEXP3-DEST and pEXP4-DEST vectors contain the necessary regulatory element in an optimal configuration for protein synthesis using the Expressway™ System. See the detailed map and feature descriptions on pages 37-40.

Bacteriophage T7 promoter for high-level, inducible expression of the recombinant protein of interest in the Expressway™ Systems or in *E. coli*

N- or C-terminal 6xHis tag for purification of recombinant fusion proteins

N- or C-terminal Lumio™ tag for specific detection of recombinant proteins using Lumio™ Technology

TEV recognition site for cleavage of the N-terminal peptide from the recombinant fusion protein using TEV protease (pEXP3-DEST only)

Two recombination sites, *att*R1 and *att*R2, downstream of the T7 promoter for recombinational cloning of the gene of interest from an entry clone

Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterscreening

*ccd*B gene located between the *att*R sites for negative selection

Ampicillin resistance gene for selection in *E. coli*

pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

E. coli slyD Extract

All Invitrogen Expressway™ System Kits contain an optimized *E. coli slyD* extract. The *slyD* extract promotes the high yield expression of full-length, active protein from DNA constructs under the reaction conditions specified in this manual.

Overview, continued

Other Expressway™ Systems

If you have used other Invitrogen Expressway™ Cell-Free *E. coli* Expression Systems, note that some of the components including the Expressway™ IVPS *E. coli* Extract and the Expressway™ 2.5X IVPS *E. coli* Reaction Buffer supplied with older Expressway™ kits contain different formulations and **may not** be compatible with this system.

For optimal results, use the components supplied in this kit to perform the protein synthesis reaction.

Lumio™ Technology

The Lumio™ System is based on the FlAsH (Fluorescein Arsenical Hairpin) technology which uses a biarsenical reagent to bind and detect proteins containing a tetracysteine motif (*i.e.*, Lumio™ tag) (Griffin *et al.*, 1998). The biarsenical reagent becomes strongly fluorescent only upon binding to the Lumio™ tag, allowing specific detection of Lumio™-tagged recombinant proteins from endogenous proteins in gels or during real-time protein synthesis. For more information about Lumio™ Technology, see page 4 and the Lumio™ Green Detection Kit manual.

Gateway® Technology

Gateway® is a universal cloning technology that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest in *E. coli* using the Gateway® Technology, simply:

Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone.

Generate an expression clone by performing an LR recombination reaction between the entry clone and pEXP3-DEST or pEXP4-DEST.

Use your expression clone in the Expressway™ *E. coli* Expression System with Lumio™ Technology for *in vitro* protein synthesis (see below).

For more information about Gateway® Technology and performing the LR recombination reaction, refer to the Gateway® Technology manual, available from www.invitrogen.com or by contacting Technical Support (page 44).

Experimental Outline

The table below describes the major steps required to synthesize your recombinant protein of interest using the Expressway™ Lumio™ Expression and Detection System. Refer to the specified pages for details to perform each step.

Step	Action	Pages
1	Generate the DNA template.	7
2	Purify your DNA template.	13
3	Perform the protein synthesis reaction.	14
Optional:	Perform real-time protein synthesis detection	17
4	Analyze tagged recombinant proteins using in-gel Lumio™ detection.	26

Lumio™ Technology

Introduction

This section provides a brief overview of Lumio™ Technology. For more information, see the Lumio™ Green Detection Kit manual included with catalog numbers K9900-70, K9900-90 and K9900-60, and available online at www.invitrogen.com.

Advantages of Lumio™ Technology

Using the Expressway™ Lumio™ Cell-Free Expression and Detection System provides the following advantages:

- Lumio™-tagged fusion protein sensitivity at subnanogram level
- Direct detection of Lumio™-tagged fusion proteins in the polyacrylamide gel without the need for staining or western blotting
- Direct, real-time monitoring of the production of Lumio™-tagged proteins during the Expressway™ *in vitro* synthesis reaction
- Detection compatible with downstream applications such as Coomassie® staining, silver staining, fluorescent staining, western blotting, or mass spectrometry analysis

Components of the Lumio™ System

The two major components of the Lumio™ System are described below:

- The tetracysteine Lumio™ tag (Cys-Cys-Pro-Gly-Cys-Cys). When fused to a gene of interest, the Lumio™ tag allows the expressed fusion protein from the pEXP Gateway® construct to be specifically recognized by the Lumio™ Green Detection Reagent. For more information on the tetracysteine motif, see below.
- A biarsenical Lumio™ Green Detection Reagent, which becomes fluorescent upon binding to recombinant proteins containing the Lumio™ tag. Lumio™ Green Detection Reagent is supplied pre-complexed to the EDT (1,2-ethanedithiol) which stabilizes and solubilizes the biarsenical reagent. For more information on how the Lumio™ Green Detection Reagent binds to the Lumio™-tag, see the Lumio™ Green Detection Kit manual.

Tetracysteine Motif

The Lumio™ Green Detection Reagent binds a tetracysteine motif consisting of Cys-Cys-Xaa-Xaa-Cys-Cys where Cys equals cysteine and Xaa equals any amino acid other than cysteine. This motif is rarely seen in naturally occurring proteins allowing specific fluorescent labeling of recombinant proteins fused to the Lumio™ tag. In the Lumio™ System, the tetracysteine motif is Cys-Cys-Pro-Gly-Cys-Cys as this motif has been shown to have a higher affinity for and more rapid binding to biarsenical compounds as well as enhanced stability compared to other characterized motifs (Adams *et al.*, 2002).

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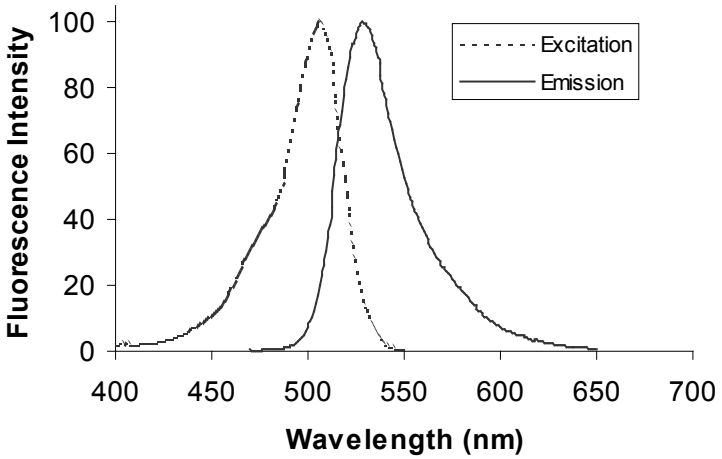
Lumio™ Technology, continued

Fluorescence Spectra

The Lumio™ Green Detection Reagent has maximum excitation at 500 nm (dye can also be excited in the UV region but with a lower efficiency) and maximum emission at 535 nm (see figure below). This allows the detection of the Lumio™ fusion proteins using a UV transilluminator equipped with a standard camera or a visible light laser-based scanner.



Comparison of Real-Time and In-Gel Lumio™ Detection Methods

Real-time Lumio™ detection allows you to directly monitor synthesis of your Lumio™-tagged recombinant proteins during the Expressway™ *in vitro* synthesis reaction. To perform real-time detection, add Lumio™ Green Detection Reagent directly to your synthesis reaction and incubate the reaction in a standard spectrofluorometer programmed to measure fluorescence at specified time points.

Note: Depending on the protein of interest, real-time detection may have varying background. We recommend that you perform in-gel Lumio™ detection immediately following real-time detection, which can be done with no loss of fluorescence intensity and without the use of additional Lumio™ Green Detection Reagent.

In-gel Lumio™ detection allows you to visualize Lumio™-tagged fusion proteins on a polyacrylamide gel. After protein synthesis, treat your Lumio™-tagged recombinant protein with the Lumio™ Green Detection Reagent and perform electrophoresis. You then image the gel using a laser-based scanner or an imager equipped with a UV transilluminator. After Lumio™ in-gel detection, the gel can be stained with Coomassie®, silver, or fluorescent stains for total protein content.

Handling the Lumio™ Green Detection Reagent

Introduction

The Lumio™ Green Detection Reagent supplied with the Lumio™ Green Detection Kit is a biarsenical compound and should be handled according to the guidelines provided in this section as well as in the Material Safety Data Sheet (MSDS).

Exercise caution when handling the Lumio™ Green Reagent. Wear protective clothing, eyewear, and gloves suitable for use with dimethyl sulfoxide (*e.g.* nitrile gloves) when handling the Lumio™ Green Detection Reagent. Review the Material Safety Data Sheet (MSDS) before handling.

Dermal Toxicity Evaluation

A dermal toxicity evaluation of the Lumio™ Green Detection Reagent was independently performed by MB Research Laboratories, Spinnerstown, PA, USA by applying a full vial of material to the mouse skin. In this study, no adverse reaction or toxicity was noted. Although arsenic compounds are toxic, this product contains <0.2% of an organic arsenic compound and shows no toxicity at a maximum dose level likely to be handled. The toxicology of this material, however, has not been fully investigated. Handle according to your chemical hygiene plan and prevent contact with this material.

Accidental Spills and Accidental Contact

Treat accidental spills of the Lumio™ Green Detection Reagent on surfaces with 10% bleach for 10 minutes and then carefully clean up. Discard arsenic-containing waste according to your institution's guidelines.

Treat accidental contact of the Lumio™ Green Detection Reagent with human skin by washing excess reagent with soap and water as soon as possible. Consult a physician following contact with Lumio™ Green Reagent. Do not treat arsenic skin exposure with EDT (1,2-ethanedithiol) as this may promote uptake of the Lumio™ Green Reagent into the body.

Disposing of the Lumio™ Green Reagent

All excess reagents that contain or have come in contact with arsenic compounds should be discarded according to your institution's guidelines and all applicable local, state, and federal requirements.

In general, we recommend disposing of protein samples labeled with the Lumio™ Green Detection Reagent and polyacrylamide gels containing protein samples labeled with the Lumio™ Green Detection Reagent as hazardous waste. For specific disposal requirements in your area, consult your safety officer.

Methods

Creating an Expression Clone

Introduction

The pEXP3-DEST vector (4.6 kb) and pEXP4-DEST vector (4.4 kb) are designed to allow T7-based, high-level expression of N- and C-terminal tagged recombinant fusion proteins using the Expressway™ Lumio™ Cell-Free Expression and Detection System. Vectors are derived from Invitrogen's pEXP1-DEST vector and adapted for use with Lumio™ Technology. For a vector map and features, see pages 37-40.

pEXP3-DEST allows you to fuse Lumio™ and 6xHis tags to the N-terminus of your protein of interest using Gateway® Technology for production of recombinant fusion proteins that can be easily detected and purified.

DNA Templates

The following DNA templates may be used in the Expressway™ Lumio™ Cell-Free Expression and Detection System:

- Supercoiled plasmid DNA (recommended to obtain the highest yields)
- Linear DNA
- PCR product

Many expression vectors or DNA templates may be used. For proper expression, all templates must contain the T7 promoter, an initiation codon, and a prokaryotic Shine-Dalgarno ribosome binding site (RBS) upstream of the gene of interest.

Generating an Entry Clone

To recombine your gene of interest into pEXP3-DEST or pEXP4-DEST, you will need an entry clone containing your gene of interest. Many entry vectors are available from Invitrogen to facilitate generation of entry clones. For more information, go to www.invitrogen.com or contact Technical Support (page 44). Refer to the manual for the specific entry vector you are using for detailed instructions to construct an entry clone.

Destination Vectors

pEXP3-DEST (supplied with K9900-70 and V960-03) allows you to fuse Lumio™ and 6xHis tags to the N-terminus of your protein of interest using Gateway® Technology. pEXP4-DEST (supplied with K9900-90 and V960-04) allows you to fuse Lumio™ and 6xHis tags to the C-terminus of your protein of interest using Gateway® Technology.

Continued on next page

Creating an Expression Clone, continued

Propagating Vectors

To propagate and maintain pEXP3-DEST and pEXP4-DEST, use 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (Catalog no. A10460) from Invitrogen. The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vector, select for transformants in media containing 50–100 µg/ml ampicillin and 15–30 µg/ml chloramphenicol.

Note: Do not use general *E. coli* cloning strains including TOP10 or DH5α for propagation and maintenance as these strains are sensitive to CcdB effects.

Points to Consider Before Recombining into pEXP3-DEST

pEXP3-DEST is an N-terminal fusion vector and contains an ATG initiation codon and a Shine-Dalgarno ribosome binding site (RBS) with optimal spacing for proper translation in *E. coli*. Your gene of interest in the entry clone must:

Be in frame with the N-terminal tag after recombination.

Contain a stop codon.

Refer to the diagram of the recombination region of pEXP3-DEST on page 9 for more information.

Points to Consider Before Recombining into pEXP4-DEST

pEXP4-DEST is an C-terminal fusion vector, your gene in the entry clone must:

Contain an ATG initiation codon and a ribosome binding site (RBS) with optimal spacing for proper translation in *E. coli*

Note: If you clone your gene of interest into an entry vector that supplies an RBS (*e.g.* pENTR/SD/D-TOPO®), then your gene of interest need only include an ATG initiation codon.

Not include a stop codon

Be in frame with the C-terminal tag after recombination.

Refer to the diagram of the recombination region of pEXP4-DEST on page 10 for more information.

Continued on next page

Generating an Expression Clone, continued

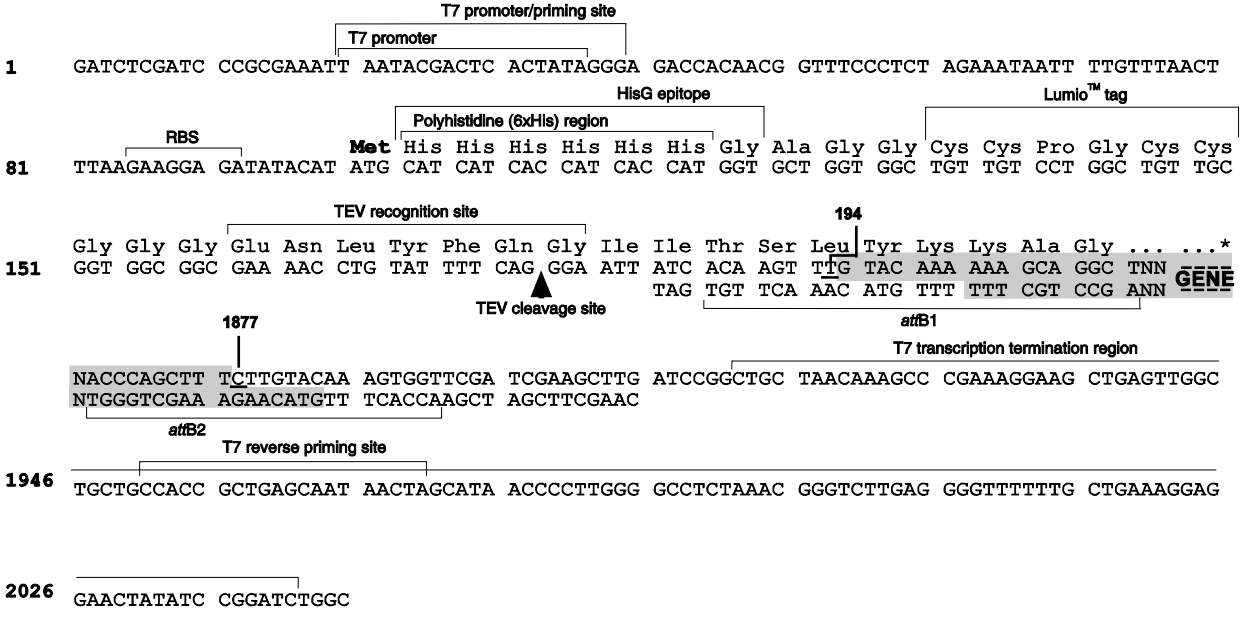
Recombination Region of pEXP3-DEST

The recombination region of the expression clone resulting from pEXP3-DEST × entry clone is shown below.

Features of the Recombination Region:

Shaded regions correspond to those DNA sequences transferred from the entry clone into the pEXP3-DEST vector by recombination. Non-shaded regions are derived from the pEXP3-DEST vector.

Bases 194 and 1877 of the pEXP3-DEST sequence are marked.



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Generating an Expression Clone, continued

Recombination Region of pEXP4-DEST

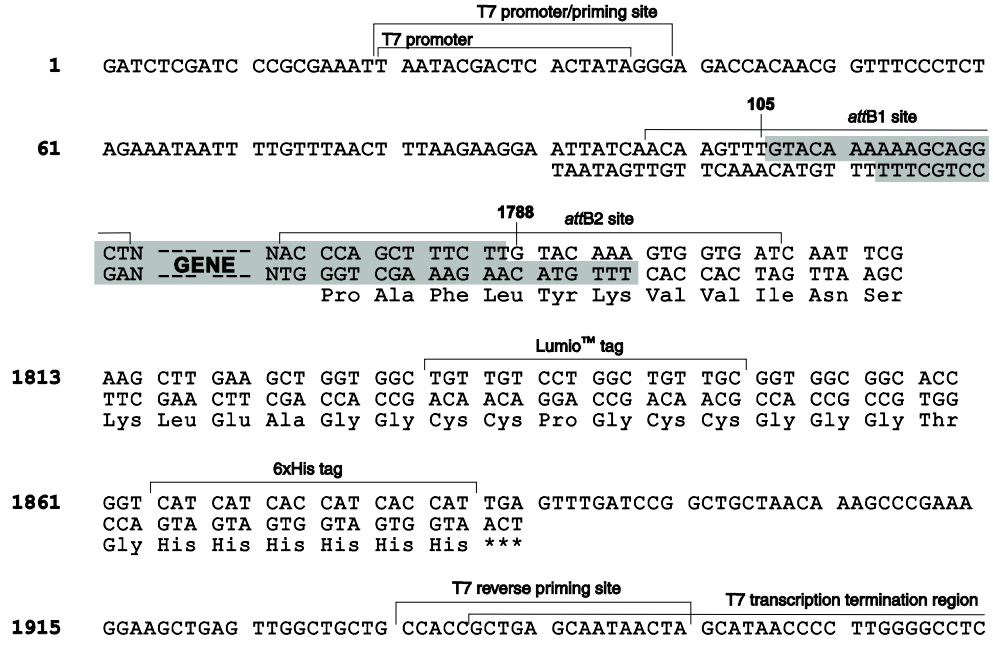
The recombination region of the expression clone resulting from pEXP4-DEST × entry clone is shown below.

Features of the Recombination Region:

Shaded regions correspond to those DNA sequences transferred from the entry clone into the pEXP4-DEST vector by recombination. Non-shaded regions are derived from the pEXP4-DEST vector.

Bases 105 and 1788 of the pEXP4-DEST sequence are marked.

A TGA stop codon is included downstream of the 6xHis tag to allow translation termination.



Performing the LR Recombination Reaction

Introduction

After you have obtained an entry clone containing your gene of interest, you will perform an LR recombination reaction between the entry clone and either pEXP3-DEST or pEXP4-DEST, and transform the reaction mixture into a suitable *E. coli* host to select for an expression clone. We recommend including the pENTR™-gus positive control supplied with the LR Clonase™ II enzyme mix in your experiments to help you evaluate your results.

E. coli Host

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α, or equivalent for transformation. **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Antibiotic Selection

Both pEXP3-DEST and pEXP4-DEST contain the ampicillin resistance gene. Expression clones may be selected using standard LB plates containing ampicillin at 100 µg/ml.

The presence of the Zeocin™ resistance gene in pEXP4-DEST allows selection of *E. coli* transformants using Zeocin™ antibiotic. For selection, use Low Salt LB agar plates containing 25 µg/ml Zeocin™ (see page 36 for a recipe). Note that for Zeocin™ to be active, the salt concentration of the bacterial medium must remain low (<90 mM) and the pH must be 7.5.

Zeocin™ is available from Invitrogen (see page vii for ordering information). Instructions to prepare and handle Zeocin™ are supplied with the product.

LR Clonase™ II Enzyme Mix

Gateway® LR Clonase™ II enzyme mix (Catalog no. 11791-020) combines the proprietary enzyme formulation and 5X LR Reaction Buffer previously supplied as separate components in Gateway® LR Clonase™ enzyme mix into an optimized single tube format to allow easier set-up of the LR recombination reaction. Use the protocol provided on the next page to perform the LR recombination reaction using LR Clonase™ II enzyme mix.

Continued on next page

Performing the LR Recombination Reaction, continued

Materials Needed

- Purified plasmid DNA of your entry clone (50-150 ng/µl in TE, pH 8.0)
- pEXP3-DEST or pEXP4-DEST (150 ng/µl in TE, pH 8.0)
- LR Clonase™ II enzyme mix (Invitrogen, Catalog no. 11791-020; keep at -20°C or -80°C until immediately before use)
- pENTR™-gus positive control, optional (50 ng/µl in TE, pH 8.0; supplied with the LR Clonase™ II enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix; thaw and keep on ice until use)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates containing the appropriate antibiotic to select for expression clones (see previous page)

Performing the LR Reaction

1. Add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix.

Component	Sample	Negative Control	Positive Control
Entry clone (50–150 ng/reaction)	1–7 µl	1–7 µl	--
pEXP3-DEST or pEXP4-DEST (150 ng/µl)	1 µl	1 µl	1 µl
pENTR™-gus (50 ng/µl)	--	--	2 µl
TE Buffer, pH 8.0	to 8 µl	to 8 µl	5 µl

2. Remove the LR Clonase™ II enzyme mix from -20°C and thaw on ice for about 2 minutes.
3. Briefly vortex the LR Clonase™ II enzyme mix twice (2 seconds each time).
4. Add 2 µl of LR Clonase™ II enzyme mix to each sample and the positive control. **Do not** add LR Clonase™ II enzyme mix to the negative control. Mix well by pipetting up and down.

   Reminder: Return LR Clonase™ II enzyme mix to -20°C or -80°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: Extending the incubation time to 18 hours typically yields more colonies.
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Transform 1 µl of the LR recombination reaction into a suitable *E. coli* host (follow the manufacturer's instructions) and select for expression clones.

   Note: You may store the LR reaction at -20°C for up to 1 week before transformation, if desired.

Continued on next page

Performing the LR Recombination Reaction, continued

What You Should See

If you use *E. coli* cells with a transformation efficiency of 1×10^8 cfu/µg, the LR reaction should give >5000 colonies if the entire LR reaction is transformed and plated.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccdB* gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/ml chloramphenicol. A true expression clone should not grow in the presence of chloramphenicol.

Sequencing

To confirm that your gene of interest is in frame with the N- or C-terminal peptide, you may sequence your expression construct using the following priming sites, if desired. Refer to the diagrams on pages 9-10 for the locations of the primer binding sites for pEXP3-DEST and pEXP4-DEST.

Vector	Forward Primer	Reverse Primer
pEXP3-DEST	T7 Promoter primer	T7 Reverse primer
pEXP4-DEST	T7 Promoter primer	T7 Reverse primer

For your convenience, Invitrogen offers a custom primer synthesis service. For more information, go to www.invitrogen.com or contact Technical Support.

Purifying the DNA Template

After you have generated the DNA template, you must purify the DNA before proceeding to the protein synthesis reaction. You may use a variety of methods to purify your DNA template including commercial DNA purification kits. For protocols to purify DNA, refer to published reference sources (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989). When purifying your DNA template, keep the following in mind:

For rapid isolation of high quality purified plasmid DNA, we recommend using the PureLink™ HQ Mini Plasmid Purification Kit available from Invitrogen. Other commercial DNA purification kits are suitable.

Do not gel-purify your DNA template. Purified DNA solution obtained from agarose gels significantly inhibits the protein synthesis reaction.

Ammonium acetate is not recommended for use in DNA precipitation as any residual contamination may inhibit translation. Use sodium acetate.

Purified DNA **must** be free of RNases (wear gloves and use RNase-free reagents when preparing DNA).

Purified DNA should be free of excess ethanol or salt as both can inhibit translation.

Note: Ethanol precipitated DNA should be carefully washed with 70% ethanol to remove excess salt and dried.

Purified DNA should be resuspended in 1X TE Buffer or water such that the final concentration is at a minimum of 500 ng/µl.

General Guidelines for Protein Synthesis

Introduction

After you have obtained purified template DNA, you are ready to synthesize recombinant protein using the Expressway™ Lumio™ Cell-Free Expression and Detection System. This section provides guidelines and a protocol to synthesize your protein.

Important

RNase contamination may affect protein yield. To reduce the chances of RNase contamination, wear gloves and use RNase-free reagents when performing the protein synthesis reaction. To eliminate RNase from surfaces, use RNase *AWAY*™ (see page vii) or a similar product.

Reaction Volumes

The volume of the protein synthesis reaction may be scaled, based on your needs. For screening reactions, the standard volume is 100 µl (50 µl initial reaction + 50 µl Feed Buffer), but this can be decreased to 25 µl reaction volume and increased up to 2 ml reaction volume. Note that protein yields may vary depending on the nature of the protein expressed and the template used.

Amount of DNA Template

For a 100 µl protein synthesis reaction, use 1 µg of template DNA (plasmid or linear DNA). For a 2 ml reaction, use 10-15 µg of template DNA. For optimal results, purify DNA template before use (see previous page).

Reaction Vessel

Use a reaction vessel that contains a large enough surface area to allow moderate mixing to occur. We recommend performing the 100 µl protein synthesis reaction in a sterile, RNase-free 1.5 ml tube. If you are performing larger reaction volumes, you may use sterile, RNase-free 50 ml conical tubes. Other reaction vessels including 96-well, 6-well or 12-well untreated culture plates are suitable.

Incubation Conditions

- To obtain optimal protein yield, **it is critical to mix the reaction thoroughly throughout the incubation period.** We recommend using a spectrofluorometer equipped with a thermomixer set to 1,200 rpm for real-time protein synthesis or a shaking incubator set to 300 rpm. **Do not** use stationary incubators such as incubator ovens or water baths as protein yields may be reduced by up to 30-50%.
- Incubate the protein synthesis reaction at a temperature ranging from 30°C to 37°C. The optimal temperature to use depends on the solubility of your recombinant protein, and should be determined empirically. Higher protein yields are generally obtained with incubation at higher temperatures (*i.e.* 37°C); however, protein solubility generally improves with incubation at lower temperatures (*i.e.* 30°C).
- You may obtain your protein of interest in as little as 1.5 hours of incubation after feeding (2 hours total). Many reactions yield 80-90% of total protein within 2 hours. However, for maximum yield, we recommend incubating the reaction for the full 6 hours.

Continued on next page

General Guidelines for Protein Synthesis, continued

Amino Acid Concentration

Use an amino acid concentration ranging from 1 mM to 4 mM in the protein synthesis reaction. The recommended amino acid concentration is 1.25 mM each, but may be adjusted according to the protein being synthesized and your application (see **Using Unnatural Amino Acids**, below).

Feed Buffer

Add 1 volume of Feed Buffer (containing Expressway™ 2X IVPS Feed Buffer and amino acids) to the protein synthesis reaction after the initial 30-minute incubation. Higher protein yields may be obtained by adding one half-volume of Feed Buffer at 30 minutes and one half-volume of Feed Buffer again at 2 hours after initiating the protein synthesis reaction.

Using Unnatural Amino Acids

Methionine is supplied separately in the kit to allow you to incorporate unnatural amino acids into your recombinant protein and adjust the amino acid concentration in the protein synthesis reaction. Depending on your application, you may use the following unnatural amino acids:

- **Radiolabeled methionine:** Use ^{35}S-Methionine to produce radiolabeled protein for use in expression and purification studies. See, pages 19 and 21 for recommended amounts of labeled and unlabeled methionine.
- **Heavy metal-labeled methionine:** Use selenomethionine (Budisa *et al.*, 1995; Doublie, 1997; Hendrickson *et al.*, 1990) to produce labeled protein for use in X-ray crystallographic studies. See pages 19 and 21 for recommended amounts of labeled methionine.

  Note: When using selenomethionine, do not use any unlabeled methioinine in the protein synthesis reaction.

Handling Reagents

- **Do not** store the *E. coli slyD*- Extract, 2.5X IVPS Reaction Buffer (-A.A.), or 2X Feed Buffer at -20°C or room temperature as this may result in loss of activity.
- Freezing and thawing the *E. coli slyD* Extract, *E. coli* Reaction Buffer (-A.A.), and 2X Feed Buffer once or twice is acceptable. However, avoid multiple freeze/thaw cycles as this may result in loss of activity.

Positive Control

The pEXP3-GW/CAT (supplied with cat nos. K9900-70, V960-03), pEXP4-ORF (supplied with cat nos. K9900-90, V960-04), or pEXP5-NT/CALML3 (supplied with cat. no. K9900-60) control vectors may be used as a positive control for protein expression. To propagate and maintain the control plasmid:

1. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™-T1^R, or equivalent. Use 10 ng of plasmid for transformation.
2. Select transformants on LB agar plates containing 100 µg/ml ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Continued on next page

General Guidelines for Protein Synthesis, continued

Choosing a Protein Synthesis Protocol

Use the table below to choose the appropriate protocol for your application needs.

If you wish to...	Then proceed to...
Generate Lumio™-tagged protein and perform real-time detection	Protein Synthesis with Real-Time Lumio™ Detection (page 19).
Generate Lumio™-tagged protein and **not** perform real-time detection	Standard Protein Synthesis (page 21).

Protein Synthesis with Real-Time Lumio™ Detection

Introduction

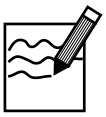
This section provides information on performing protein synthesis with real-time detection of Lumio™-tagged proteins. Note that real-time signal strength does not correlate to protein expression levels, so performing in-gel detection is recommended in addition to real-time detection.

Real-Time Lumio™ Detection

Real-time detection of Lumio™-tagged proteins allows you to directly monitor production of your recombinant proteins during protein synthesis using a standard spectrofluorometer. To perform real-time detection, simply add Lumio™ Green Detection Reagent to your protein synthesis reaction and incubate the reaction in a spectrofluorometer programmed to measure fluorescence at specified time points.

Important

Real-time detection of Lumio™-tagged proteins is **NOT** a direct quantitative method to determine protein concentration. Depending on the folding conformation of the protein, the Lumio™ tag may be buried within folded regions thereby preventing binding of the detection reagent, or the expressed protein itself may quench the fluorescence. The fluorescent signal generated does not necessarily correspond to the amount of protein synthesized, and should be confirmed using in-gel detection (page 24) following real-time analysis.



Note

You can perform in-gel Lumio™ detection following real-time detection with no loss of fluorescent intensity and without the use of additional Lumio™ Green Detection Reagent. Refer to the section on in-gel Lumio™ detection on page 24 for instructions on preparing for in-gel detection following real-time analysis.

Protein Synthesis with Real-Time Lumio™ Detection, continued

Materials Needed

Provided by the user:

- Expression construct or other suitable DNA template (purified; resuspended in TE or water at a concentration greater than 500 ng/µl)

^{35}S Methionine, optional (3,000 Ci/mmol; 15 µCi/µl)

Tubes or microplates suitable for use with your spectrofluorometer

Spectrofluorometer equipped with an incubator and mixer (see next page)

RNase-free pipette tips and microcentrifuge tubes

Supplied with the kit:

Expressway™ *E. coli slyD*- Extract (thaw on ice)

Expressway™ 2.5X IVPS Reaction Buffer (-A.A.) (thaw on ice)

Expressway™ 2X IVPS Feed Buffer (thaw on ice)

T7 Enzyme Mix (keep on ice; store at -20°C after initial use)

50 mM Amino Acids (-Met)

Note: When thawing the 50 mM Amino Acids (-Met), the solution may have a brown or yellowish tint. This is normal and does not affect the activity of the amino acids.

75 mM Methionine

DNase/RNase-free distilled water

- pEXP3-GW/CAT or pEXP4-ORF control plasmid, optional; resuspended to 0.5 µg/µl in sterile water

  Note: The pEXP5-NT/CALML3 control plasmid (supplied with cat no. K9900-60) is not a Lumio™ fusion vector and should not be used as a control for real-time Lumio™ detection.

- Lumio™ Green Detection Reagent

  Note: The color of the Lumio™ Green Detection Reagent may change from clear to pink during storage. This will not affect the performance of the reagent.

Protein Synthesis with Real-Time Lumio™ Detection, continued

Instrument Specifications

For real-time detection of Lumio™-tagged proteins, we recommend performing the protein synthesis reaction in a spectrofluorometer with a built-in incubator and mixer (*e.g.*, Molecular Devices Gemini XS Spectrofluorometer). The wavelength settings for Lumio™ detection are:

Excitation wavelength: 500 nm

Emission wavelength: 535 nm

Additional fluorescence spectra information is provided on page 5.

If your spectrofluorometer is not equipped with an incubator and/or mixer, you can incubate the reaction in a thermomixer or shaking incubator/water bath and transfer the tubes/plates to the spectrofluorometer at regular intervals during the reaction to perform the reading (see **Alternative**, next page). We do not recommend using a non-shaking incubator because it produces a less stable and less consistent temperature environment.

Performing Protein Synthesis with Real-Time Lumio™ Detection

Use the protocol below to synthesize your protein from the DNA template with real-time detection of Lumio™-tagged proteins.

1. Thaw the Lumio™ Green Detection Reagent and mix well by pipetting up and down.
2. Prepare reactions in tubes or microplates suitable for use with your spectrofluorometer. For each sample, add the following reagents to each tube or well on ice. For multiple samples, scale up the volume of each reagent accordingly and aliquot the cocktail into individual tubes/wells.

Reagent	Amount
E. coli slyD- Extract	20 µl
2.5X IVPS Reaction Buffer (-A.A.)	20 µl
50 mM Amino Acids (-Met)	1.25 µl – 4 µl
75 mM Methionine*	1 µl – 3 µl
T7 Enzyme Mix	1 µl
DNA Template	1 µg
Lumio™ Green Detection Reagent	1 µl
DNase/RNase-free Distilled Water	To a final volume of 50 µl

***Note:** To generate radiolabeled protein using ^{35}S-methionine, use 2 µl of ^{35}S-methionine, and 1 µl unlabeled 75 mM methionine. To generate labeled protein using selenomethionine, use 2 µl of selenomethionine only, do not add unlabeled methionine.

3. Program your spectrofluorometer for a 2-hour (up to 6-hour) incubation at 37°C with mixing. Program fluorescence data collection at specified time points (*e.g.*, every 10 minutes). See above for wavelength settings for Lumio™ detection.

Protocol continues on next page

Continued on next page

Protein Synthesis with Real-Time Lumio™ Detection, continued

Performing Protein Synthesis with Real-Time Lumio™ Detection (continued)

Continued from the previous page

4. After programming the spectrofluorometer, insert your sealed tubes or microplate and run the program.

   Alternative: If your spectrofluorometer does not include an incubator or mixer, you can incubate the tubes or microplate at 37°C for 2 hours in a thermomixer set at 14,000 rpm or shaking incubator set at 275–325 rpm. At regular intervals (*e.g.*, 10 minutes), transfer the tubes/microplate to a spectrofluorometer and collect excitation and emission data.

5. Let the program run for 30 minutes, and during this time, prepare the Feed Buffer. **For each sample**, add the following reagents to a sterile, RNase-free microcentrifuge tube. For multiple samples, you may scale up the volume of reagents used accordingly and prepare one master mix.

Reagent	Amount
2X IVPS Feed Buffer	25 µl
50 mM Amino Acids (-Met)	1.25 µl – 4 µl
75 mM Methionine*	1 µl - 3µl
Lumio™ Green Detection Reagent	1 µl
DNase/RNase-free Distilled Water	To final volume of 50 µl

   ***Note:** To generate radiolabeled protein using ^{35}S-methionine, use 2 µl of ^{35}S-methionine, and 1 µl unlabeled 75 mM methionine. To generate labeled protein using selenomethionine, use 2 µl of selenomethionine only, do not add unlabeled methionine.

6. After 30 minutes of incubation (from Step 5 above), add 50 µl of the Feed Buffer to the samples (total volume = 100 µl) between data time points.

   Note: You may incubate the reaction for up to 6 hours to obtain greater protein yield. You may also incubate at temperatures as low as 25°C to decrease the rate of protein synthesis and to promote proper folding. If you will be incubating tubes at temperatures lower than 37°C, we recommend extending the incubation time to 4 hours.

To prepare proteins for in-gel Lumio™ detection briefly centrifuge and place the reaction on ice. Proceed to **Performing In-Gel Lumio™ Detection**, page 23.

Standard Protein Synthesis

Introduction

This section provides information on performing a standard Expressway™ Cell-free *E. coli* protein synthesis reaction without real-time detection of Lumio™-tagged fusion proteins. You can detect your Lumio™-tagged fusion proteins using in-gel Lumio™ detection (see page 23) following standard protein synthesis.

Incubation Conditions

We recommend using an Eppendorf Thermomixer (Fisher, Catalog no. 05-400-200) to shake your sample(s) at 37°C during the protein synthesis reaction. If a thermomixer is unavailable, you may use a standard shaking incubator or a standard shaking water bath. We do not recommend using a non-shaking incubator because it produces a less stable and less consistent temperature environment.

Materials to Have on Hand

Provided by the user:

- Expression construct or other suitable DNA template (purified; resuspended in TE or water at a concentration greater than 500 ng/µl)

^{35}S Methionine, optional (3,000 Ci/mmol; 15 µCi/µl)

Tubes or microplates suitable for use with your spectrofluorometer

Thermomixer or standard shaking incubator (see above)

RNase-free pipette tips and microcentrifuge tubes

Supplied with the kit:

Expressway™ *E. coli slyD-* Extract (thaw on ice)

Expressway™ 2.5X IVPS Reaction Buffer (-A.A.) (thaw on ice)

Expressway™ 2X IVPS Feed Buffer (thaw on ice)

T7 Enzyme Mix (keep on ice; store at -20°C after initial use)

50 mM Amino Acids (-Met)

Note: When thawing the 50 mM Amino Acids (-Met), the solution may have a brown or yellowish tint. This is normal and does not affect the activity of the amino acids.

75 mM Methionine

DNase/RNase-free distilled water

- Control plasmid, optional; resuspended to 0.5 µg/µl in sterile water

Continued on next page

Standard Protein Synthesis, continued

Performing Standard Protein Synthesis

Use the protocol below to synthesize your protein from the DNA template *without* real-time Lumio™ detection.

1. **For each sample**, add the following reagents to the appropriate reaction vessel on ice. For multiple samples, scale up the volume of each reagent accordingly and aliquot the cocktail into individual tubes/wells.

Reagent	Amount
E. coli slyD- Extract	20 µl
2.5X IVPS Reaction Buffer (-A.A.)	20 µl
50 mM Amino Acids (-Met)	1.25 µl – 4 µl
75 mM Methionine*	1 µl – 3 µl
T7 Enzyme Mix	1 µl
DNA Template	1 µg
DNase/RNase-free Distilled Water	To a final volume of 50 µl

***Note:** To generate radiolabeled protein using ^{35}S-methionine, use 2 µl of ^{35}S-methionine, and 1µl unlabeled 75 mM methionine. To generate labeled protein using selenomethionine, use 2 µl of selenomethionine only, do not add unlabeled methionine.

2. Close the tube and incubate sample in a standard shaking incubator (300 rpm) at 30°C for 30 minutes. If the protein you are synthesizing is known to be soluble, you may incubate the sample at 37°C.
3. During the 30-minute incubation, prepare the Feed Buffer. **For each sample**, add the following reagents to a sterile, RNase-free microcentrifuge tube. For multiple samples, you may scale up the volume of reagents used accordingly and prepare one master mix.

Reagent	Amount
2X IVPS Feed Buffer	25 µl
50 mM Amino Acids (-Met)	1.25 µl – 4 µl
75 mM Methionine*	1 µl – 3 µl
DNase/RNase-free Distilled Water	To final volume of 50 µl

***Note:** To generate radiolabeled protein using ^{35}S-methionine, use 2 µl of ^{35}S-methionine, and 1 µl unlabeled 75 mM methionine. To generate labeled protein using selenomethionine, use 2 µl of selenomethionine only, do not add unlabeled methionine.

4. After 30 minutes of incubation (from Step 2 above), add 50 µl of the Feed Buffer to the sample (total volume = 100 µl).
5. Cap the tube and return the sample to the shaking incubator (300 rpm). Incubate for up to 6 hours at 30-37°C as appropriate (see page 14).

To prepare proteins for in-gel Lumio™ detection centrifuge and place the reaction on ice. Proceed to **Performing In-Gel Lumio™ Detection**, next page.

Performing In-Gel Lumio™ Detection

Introduction

After you have synthesized your protein, you are ready to prepare the protein samples for analysis with the Lumio™ Green Detection Kit. To detect Lumio™-tagged fusion proteins by polyacrylamide gel electrophoresis, you must first precipitate the proteins with acetone to remove background smearing and add the Lumio™ Gel Sample Buffer and Lumio™ In-Gel Detection Enhancer. If you have not already added Lumio™ Green Detection Reagent for real-time detection, you will also add this reagent. General guidelines and protocols are provided below. For more detailed information, refer to the Lumio™ Green Detection Kit manual.

If you have performed trace labeling using ^{35}S-Methionine, you may use TCA precipitation to determine the amount of radiolabeled methionine incorporated and to calculate the yield of protein (see **Determining Protein Yield**, page 28).

Recommended Gels

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. For more information about pre-cast gels available go to www.invitrogen.com or contact Technical Support (see page 44).

BenchMark™ Fluorescent Protein Standard

The BenchMark™ Fluorescent Protein Standard (also available separately as cat. no. LC5928) allows easy and direct visualization of molecular weight ranges of your Lumio™ fusion protein on a SDS-PAGE gel. For detailed information and specifications, refer to the BenchMark™ Fluorescent Protein Standard manual.

The BenchMark™ Fluorescent Protein Standard proteins are easily detected using a UV transilluminator or a visible light laser-based scanner at the same excitation and emission wavelengths as your Lumio™ fusion protein. The standard consists of seven distinct protein bands in the range of ~11-155 kDa and is supplied in a ready-to-use format. The recommended loading volume is 5 µl.

Lumio™ Gel Sample Buffer

The Lumio™ Gel Sample Buffer (4X) supplied with the kit is a proprietary sample buffer containing protein denaturing and reducing agents. The buffer is specifically formulated to provide optimal results with the Lumio™ Green Detection Reagent. **Always use the Lumio™ Gel Sample Buffer (4X) to prepare samples for electrophoresis.**

To prevent oxidation of the reducing agent in the buffer, store the Lumio™ Gel Sample Buffer (4X) at -20°C and minimize exposure to air. Use the buffer immediately upon removal from -20°C and return the buffer to -20°C immediately after use.

Lumio™ In-Gel Detection Enhancer

The Lumio™ In-Gel Detection Enhancer is a proprietary solution and is designed to reduce the non-specific binding of Lumio™ Green Detection Reagent with endogenous proteins.

Continued on next page

Performing In-Gel Lumio™ Detection, continued

For optimal results with the Lumio™ Green Detection Kit, follow these guidelines:

- Load at least 1 picomole of the Lumio™ fusion protein
- Use 5 µl of BenchMark™ Fluorescent Protein Standard on a mini-gel as a molecular weight marker
- **Always use the Lumio™ Gel Sample Buffer (4X) to prepare samples for electrophoresis**
- Wear protective clothing, eyewear, and gloves suitable for use with dimethyl sulfoxide (*e.g.*, nitrile gloves) when handling the Lumio™ Green Detection Reagent
- Use the Lumio™ Gel Sample Buffer (4X) in a certified fume hood
- Use an appropriate percentage of acrylamide gel that will best resolve your protein of interest
- Visualize the gel immediately after electrophoresis to prevent diffusion of proteins as the proteins are not fixed in the gel during Lumio™ detection
- Avoid touching the gel with bare hands while handling or imaging the gel
- Proteins that DO NOT contain Lumio™ Green Detection Reagent may be stored in 1X Lumio™ Gel Sample Buffer at -20°C.

Materials Needed

- Protein synthesis reaction from page 19 or page 22
- Acetone at -20°C
- 4X Lumio™ Gel Sample Buffer (supplied with the kit)
- Lumio™ Green Detection Reagent (if not already added for real-time detection; supplied with the kit)
- Lumio™ In-Gel Detection Enhancer (supplied with the kit)
- Water bath or heat block set at 70°C
- Freezer set at -20°C
- Appropriate pre-cast gels and running buffer

Preparing Expressed Proteins

Use the following protocol to prepare your proteins for gel electrophoresis.

1. To 5 µl of the protein synthesis reaction product from page 19 or page 22, add 20 µl of cold acetone (-20°C). Mix well.
2. Incubate at -20°C for 20 minutes.
3. Centrifuge for 5 minutes at room temperature in a microcentrifuge at 12,000 rpm.
4. Carefully remove the supernatant without disturbing the protein pellet. Air dry for 2-3 minutes to allow excess acetone to evaporate.
5. Dilute the 4X Lumio™ Gel Sample Buffer included with the kit to 1X with deionized water.
6. Resuspend the pellet from Step 3 in 20 µl of 1X Lumio™ Gel Sample Buffer.
7. Proceed to **Adding Lumio™ Detection Reagent**, next page.

Continued on next page

Performing In-Gel Lumio™ Detection, continued

Adding Lumio™ Detection Reagent

Add Lumio™ Detection Reagent to your protein sample as described below for in-gel detection.

Important: If you have already added Lumio™ Detection Reagent to your protein sample for real-time detection (page 19), **skip this procedure** and proceed directly to **Adding Lumio™ In-Gel Detection Enhancer**, below.

1. Thaw the Lumio™ Green Detection Reagent and mix well by pipetting up and down.
2. Add 0.2 µl of the Lumio™ Green Detection Reagent to each sample using a 2 µl pipetter (*e.g.*, a P2 pipetter). Return the Lumio™ Green Detection Reagent to -20°C immediately after use.

   Alternative: If you do not have a P2 pipetter, make a 1:5 dilution of the Lumio™ Green Detection Reagent using 1X Lumio™ Gel Sample Buffer. Add 1 µl of this diluted Lumio™ Green Detection Reagent to each sample. Diluted Lumio™ Green Detection Reagent is stable for up to 1 week at -20ºC.
3. Mix samples well by pipetting up and down and incubate samples at 70°C for 10 minutes.
4. Allow samples to cool for 1-2 minutes and centrifuge briefly at high speed in a microcentrifuge.
5. Proceed to **Adding Lumio™ In-Gel Detection Enhancer**, below.

Adding Lumio™ In-Gel Detection Enhancer

Add Lumio™ In-Gel Detection Enhancer as described below:

1. Thaw the Lumio™ In-Gel Detection Enhancer and mix well by pipetting up and down.
2. Add 2 µl of Lumio™ Gel Detection Enhancer to each protein sample.
3. Mix samples well by pipetting up and down and incubate samples at room temperature for 5 minutes. Return the Lumio™ In-Gel Detection Enhancer to -20°C immediately after use.
4. Load 5–20 µl of each sample on an appropriate gel and perform electrophoresis. Be sure to run the gel long enough so that the dye front runs off the bottom of the gel, to avoid masking smaller proteins.
5. Proceed to **Analyzing Lumio™-Tagged Proteins in Gels**, next page.

Important

After the Lumio™ Green Detection Reagent and the Lumio™ Gel Detection Enhancer have been added to sample, proceed to visualizing proteins within a few hours to avoid photobleaching.

Analyzing Lumio™-Tagged Proteins in Gels

Introduction

After you have performed electrophoresis, you can visualize Lumio™-tagged recombinant proteins directly in the gel. General guidelines are provided below. For more detailed information, refer to the Lumio™ Green Detection Kit manual.

Important

Detection of recombinant proteins with the Lumio™ Green Detection Kit is **not permanent** and is lost by subsequent staining of the gel with other protein stains and western blotting. It is extremely important to record a permanent image of the gel prior to staining the gel with protein stains and gel drying.

Imager Specifications

For optimal visualization of the fluorescent protein bands after detection with Lumio™ Green Detection Kit, you will need:

- An imager equipped with a UV transilluminator (302 or 365 nm); a standard camera or CCD camera; and an ethidium bromide filter, SYBR® Green filter, or band pass filter encompassing the emission maxima (535 nm) of the stain.

  Note: If you are using a 365 nm UV transilluminator, you may have to expose the gel for a longer time, as the sensitivity is lower than a 302 nm UV transilluminator.

  OR

- A laser-based scanner with a laser line that falls within the excitation maxima of the stain (500 nm), a 535 nm long pass filter, or a band pass filter center near the emission maxima of 535 nm. The sensitivity of detection is more with laser-based scanners equipped with appropriate filters than with UV transillumination.

- If you are using pre-cast gels in cassettes, and are performing imaging with a UV-transilluminator, we recommend removing the gel from the cassette after electrophoresis is complete. Avoid touching the gel with bare hands while handling or imaging the gel.
- If you are imaging with a laser-based scanner, you do not have to remove the gel from the cassette.

UV Exposure Time

If you are using an imager with UV transilluminator, be sure to adjust the settings and filters on the imager **prior to turning on the UV light**. The fluorescent dye of the Lumio™ Green Reagent is sensitive to photobleaching. We recommend a 4-10 second exposure. Avoid exposing the gel to UV light for a long time.

You should see fluorescent bands of Lumio™ fusion proteins and the gel should have minimal background.

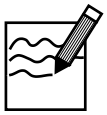
Note: The Lumio™ fusion protein bands appear white or black depending on the type of imaging system used for imaging the gels.

Continued on next page

Analyzing Lumio™-Tagged Proteins in Gels, continued

Assay for CAT

If you use the pEXP3-GW/CAT positive control vector, you can assay for CAT protein using CAT Antiserum available from Invitrogen (see page vii for ordering information). Other commercial kits are available for assaying CAT expression. The molecular weight of the CAT fusion protein is approximately 30 kDa.



Note

The N- and C-terminal peptide containing the Lumio™ tag and 6xHis tag will add approximately 3.5 kDa to your protein.

What to Do Next

Once you have verified expression, you may use the recombinant protein in any downstream application of your choice. If you plan to use the recombinant protein for structural analyses including x-ray crystallography, note that you must purify the recombinant protein before use. Use any method of choice to purify your recombinant protein.

If you have expressed your recombinant protein with the N-terminal or C-terminal 6xHis tag, you may purify your recombinant protein using a metal-chelating resin such as ProBond™ or Ni-NTA. For guidelines to purify recombinant protein using ProBond™ or Ni-NTA, see page viii.

Note: Other metal-chelating resins are suitable.

Determining Protein Yield

Introduction

If you have included radiolabeled methionine in the protein synthesis reaction, you may use TCA precipitation to determine the amount of radiolabeled methionine incorporated and to calculate the yield of protein.

Determining Total Counts

1. Mix and spot 5 µl of each radiolabeled reaction from page 19 or page 22 on a glass microfiber filter (Type GF/C; Whatman, Catalog no. 1822-021).
2. Set aside and let dry. **Do not** wash or TCA precipitate these filters.
3. Place the filters in scintillation vials, and add scintillation fluid. Count samples in a scintillation counter.

Performing TCA Precipitation

A protocol is provided below to perform TCA precipitation using a vacuum filtration device (*e.g.* Millipore 1225 Sampling Manifold or similar).

Performing TCA Precipitation Using a Vacuum Filtration Device

1. Aliquot 5 µl of each radiolabeled reaction from page 19 or page 22 into separate glass tubes.
2. Add 100 µl of 1 N NaOH to each reaction and incubate for 5 minutes at room temperature.
3. Add 3 ml of 10% TCA to each glass tube and incubate tubes at +4ºC for 20 minutes.
4. Wet individual glass fiber (GF/C) filters with 10% TCA and place onto the vacuum filtration device.
5. Turn the vacuum on and pour the TCA solution from each glass tube into a sample well.
6. Wash filters twice with 5% TCA.
7. Wash filters once with 100% ethanol. Leave the vacuum on for 1 minute to allow the filters to dry.
8. Turn the vacuum off and remove the filters. Place the filters in scintillation vials, and add scintillation fluid. Count samples in a scintillation counter.
9. Proceed to **Calculating Protein Yield**, next page.

Continued on next page

Determining Protein Yield, continued

Calculating Protein Yield

Use the equations below to calculate the yield of protein obtained. You will need to determine the pmoles of methionine present in your specific reaction. Remember to account for both radiolabeled and unlabeled methionine. You will also need to determine the total counts incorporated using TCA precipitation (see previous page).

Total counts:

$$\text{total cpm per 5 } \mu\text{l spotted} \times \frac{\text{total reaction volume}}{5}$$

Specific activity:

$$\frac{\text{total counts}}{\text{pmoles of methionine}}$$

pmoles methionine incorporated:

$$\frac{\left[(\text{TCA precipitable counts} - \text{background}) \times \frac{\text{total reaction volume}}{5}\right]}{\text{specific activity}}$$

pmoles of protein:

$$\frac{\text{pmoles of methionine incorporated into protein}}{\text{number of methionines in protein}}$$

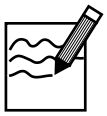
Yield of protein (in μg):

$$\frac{\text{moles of protein} \times \text{molecular weight of protein}}{10^6}$$

Purifying the Recombinant Soluble Fusion Protein

Introduction

The presence of the N-terminal or C-terminal 6xHis tag in the pEXP3-DEST, and pEXP4-DEST vectors allows purification of your recombinant protein with a metal-chelating resin such as ProBond™ or Ni-NTA (see page viii for ordering information). This section provides guidelines for purification.



Note

The pEXP3-DEST vector contains a Tobacco Etch Virus (TEV) recognition site to allow removal of the N-terminal tag from your recombinant fusion protein using TEV protease. See page vii for additional products.

ProBond™ and Ni-NTA

ProBond™ and Ni-NTA are nickel-charged agarose resins that can be used for affinity purification of fusion proteins containing the 6xHis tag. Proteins bound to the resin may be eluted with either low pH buffer or competition with imidazole or histidine.

- To purify your fusion protein using ProBond™ or Ni-NTA, follow the guidelines below and detailed instructions included with each product. You may download the appropriate manuals from www.invitrogen.com.
- To purify your fusion protein using another metal-chelating resin, refer to the manufacturer's instructions.

Guidelines for Purification

Follow these guidelines when purifying your recombinant fusion protein using ProBond™ or Ni-NTA. Remember to use criteria appropriate for purification under native conditions. For details, refer to the ProBond™ or Ni-NTA manual, as appropriate.

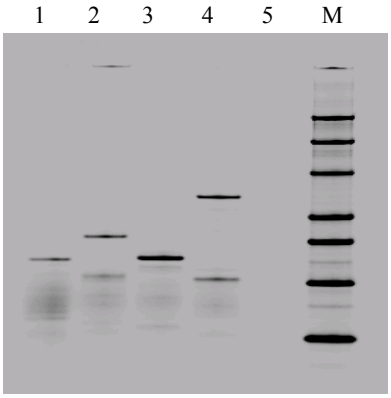
1. Prepare the purification column containing ProBond™ or Ni-NTA agarose resin. After applying the resin to the purification column, wash with 4 volumes of water followed by 8 volumes of Binding Buffer (supplied with the kit; 50 mM $NaPO_4$, pH 8.0, 500 mM NaCl) to equilibrate the column.
2. **Optional (applies only to protein synthesis reactions containing extra components (*e.g.* detergents, chaperones) other than those supplied with the Expressway™ kit):** Dilute the Expressway™ reaction (from page 19 or page 22) 1:1 with Binding Buffer (50 mM $NaPO_4$, pH 8.0, 500 mM NaCl).
3. Centrifuge the reaction at 15,000 x g for 10 minutes at room temperature to remove insoluble material.
4. Load the supernatant containing soluble protein onto the equilibrated resin and incubate (*i.e.* batch binding) for 30 minutes at the desired temperature.
5. Wash the column twice with 2 volumes of Binding Buffer each time.
6. Wash the column twice with 2 volumes of Binding Buffer containing 20 mM imidazole.
7. Elute the protein using an Elution buffer containing an appropriate amount of imidazole (*e.g.* 250 mM imidazole).
8. Analyze the fractions using SDS-PAGE.
9. Pool the desired fractions and dialyze, if necessary.

Sample Protein Synthesis Experiment

Introduction

This section provides an example of a typical protein synthesis experiment performed using the Expressway™ Lumio™ Cell-Free Expression and Detection System. Results from sample protein synthesis reactions using pEXP3-DEST with in-gel and real-time Lumio™ detection are provided below. Note that real-time signal strength does not correlate to protein expression levels, so performing in-gel detection is recommended in addition to real-time detection,

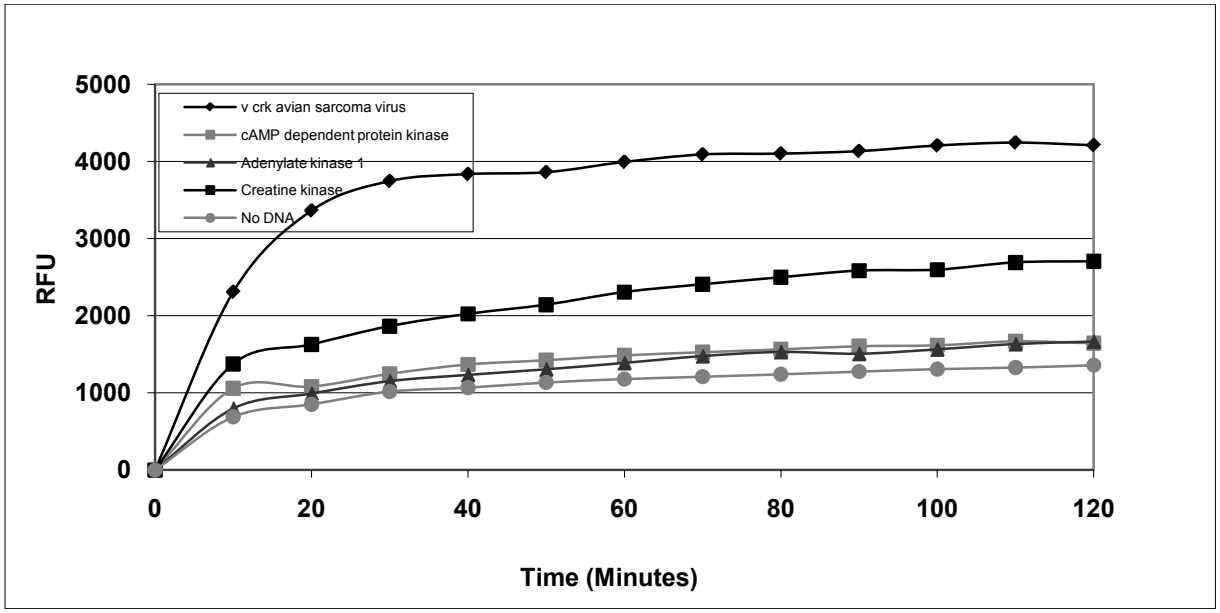
Figure 1: In-gel detection of expressed proteins containing the Lumio™ sequence



1 µl of each protein synthesis reaction was run on a 4-12% NuPAGE® gradient gel and visualized using a Typhoon laser scanner.

Lane 1: v-crk avian sarcoma virus
Lane 2: cAMP-dependent protein kinase
Lane 3: adenylate kinase
Lane 4: creatine kinase
Lane 5: No DNA control
Lane M: BenchMark™ Pre-Stained Protein Ladder

Figure 2: Real-Time incorporation of the Lumio™ tag in human ORFs



Troubleshooting

Introduction

Review the information in this section to troubleshoot your cell-free expression experiment. See the Lumio™ Green Detection Kit manual for troubleshooting Lumio™ Technology.

Synthesizing Proteins

The table below lists some potential problems and possible solutions that may help you troubleshoot your protein synthesis experiments.

Problem	Reason	Solution
Low or no yield of protein (but control reaction produces protein)	DNA template not optimally configured	• Make sure that the ATG initiation codon is in the proper context for expression (*i.e.* check spacing and placement after the RBS). • Fusion of your protein to an N- or C-terminal tag may affect RNA structure and lower translation levels. Try moving the fusion tag to the other terminus using either pEXP3-DEST or pEXP4-DEST.
	Gene of interest not cloned in frame with the N- or C-terminal tag	Generate a new expression construct, making sure that your gene of interest is cloned in frame with the N- or C-terminal tag; confirm by sequencing.
	Incorrect LR recombination reaction protocol used	The LR reaction protocol provided on page 12 is for use with **LR Clonase™ II enzyme mix only**. If you are using LR Clonase™ enzyme mix, refer to the protocol provided with the product.
	DNA template not pure • Contaminated with ethanol, sodium salt, or ammonium acetate • Contaminated with RNases	• Prepare new DNA template taking care to remove excess ethanol and/or salt after precipitation. • Do not use ammonium acetate to precipitate DNA. Use sodium acetate. • Wear gloves and use RNase-free reagents when preparing DNA.
	DNA template purified from agarose gel	Do **not** purify your DNA from a gel. See the purification guidelines on page 13
	Insufficient amount of DNA template used	• Use 10-15 µg of template DNA in a 2 ml protein synthesis reaction. • If you are expressing a large protein, increase the amount of DNA template used in the protein synthesis reaction to 20 µg.

Continued on next page

Troubleshooting, continued

Synthesizing Proteins, continued

Problem	Reason	Solution
Low or no yield of protein (but control reaction produces protein), continued	Sample incubated in a non-shaking incubator or spectrofluorometer without a mixer	• If you are not performing real-time Lumio™ detection, use a thermomixer (1,400 rpm) or a shaking incubator (275-325 rpm) or a water bath (see page 21). • For real-time Lumio™ detection, use a spectrofluorometer with an incubator and mixer or use a recommended alternative (page 19).
	Insufficient feeding	• Add one volume of Feed Buffer to the sample (*i.e.* 1 ml Feed Buffer to 1 ml sample) 30 minutes after initiating protein synthesis. Add one-half volume of Feed Buffer to the sample (*i.e.* 25 µl Feed Buffer to 50 µl sample) 30 minutes and 2 hours after initiating protein synthesis.
	Large protein being expressed	• Protein yield may decrease as the size of the protein increases; optimize expression conditions. • Reduce incubation temperature to 25°C-30°C during protein synthesis.
	Expression time too short	Extend expression time up to 4 hours.
	Protein forms aggregates	Reduce the incubation temperature to 25°C-30°C during protein synthesis. Add mild detergents (*e.g.* up to 0.05% Triton-X-100, 0.025% sodium dodecyl maltoside, 0.1% CHAPS, or 0.05% Brij-58) to the reaction and Feed Buffer. Add molecular chaperones to the reaction.
	Sample not mixed before spotting on filter for TCA precipitation (radiolabeled samples only)	Mix sample before spotting on filter for TCA precipitation.

Continued on next page

Troubleshooting, continued

Synthesizing Proteins, continued

Problem	Reason	Solution
Control reaction produces no protein	Reagents have lost activity	Store reagents at -80°C. Store the T7 Enzyme Mix at -20°C after initial use. • Use care when freezing and thawing the Expressway™ *E. coli slyD-* Extract, Expressway™ 2.5X IVPS Reaction Buffer, and Expressway™ 2X IVPS Feed Buffer. Follow handling guidelines on page 15. One or two freeze/thaw cycles are acceptable. Avoid multiple freeze/thaw cycles.
	Reagent(s) contaminated with RNases	Wear gloves and use RNase-free supplies when handling the reagents supplied in the kit. Use RNase *AWAY*™ available from Invitrogen (page vii) to eliminate RNase from surfaces.
Protein has low biological activity	Improper protein folding	Reduce incubation temperature to as low as 25°C during protein synthesis.
	Post-translational modifications required	The Expressway™ *E. coli slyD-* Extract will not introduce post-translational modifications such as phosphorylation or glycosylation to the recombinant protein.
	Synthesized protein requires co-factors for complete activity	Add required co-factors to the protein synthesis reaction.

Continued on next page

Troubleshooting, continued

Analyzing Proteins

The table below lists some potential problems and possible solutions that may help you troubleshoot your electrophoresis experiments.

Problem	Reason	Solution
Multiple bands observed on the polyacrylamide gel	Proteins denatured for too long	Add 1X SDS-PAGE sample buffer to the sample and incubate at 70°C-80°C for 10-15 minutes before loading on the gel.
	Old ^{35}S-Methionine used (radiolabeled samples only)	Use fresh ^{35}S-Methionine.
	Internal ATG codons in the context of RBS-like sequences	• Check the sequence of your gene and search for potential RBSs with the proper spacing from internal methionines. • Replace the methionine or change RBS sequence(s) using point mutation(s).
Smearing on the gel	Samples not precipitated with acetone	Precipitate the proteins with acetone to remove background smearing. Follow the protocol provided on page 24.
	Too much protein loaded	Reduce the amount of protein loaded on the gel.
	Gel not clean	Rinse the gel briefly before exposing to film. If you have stained the gel with Coomassie blue, destain the gel in water or 50% methanol, 7.5% glacial acetic acid for 15-30 minutes before drying. If you have already destained the gel, repeat destaining procedure.
	Ethanol present in the protein synthesis reaction	Make sure that any residual ethanol is removed during DNA purification.
	Old pre-cast gels	Do not use pre-cast gels after the expiration date.

Lumio™ Detection

To troubleshoot Lumio™ Detection, refer to the manual supplied with the kits, or available from www.invitrogen.com.

Appendix

Recipes

Low Salt LB Medium with Zeocin™

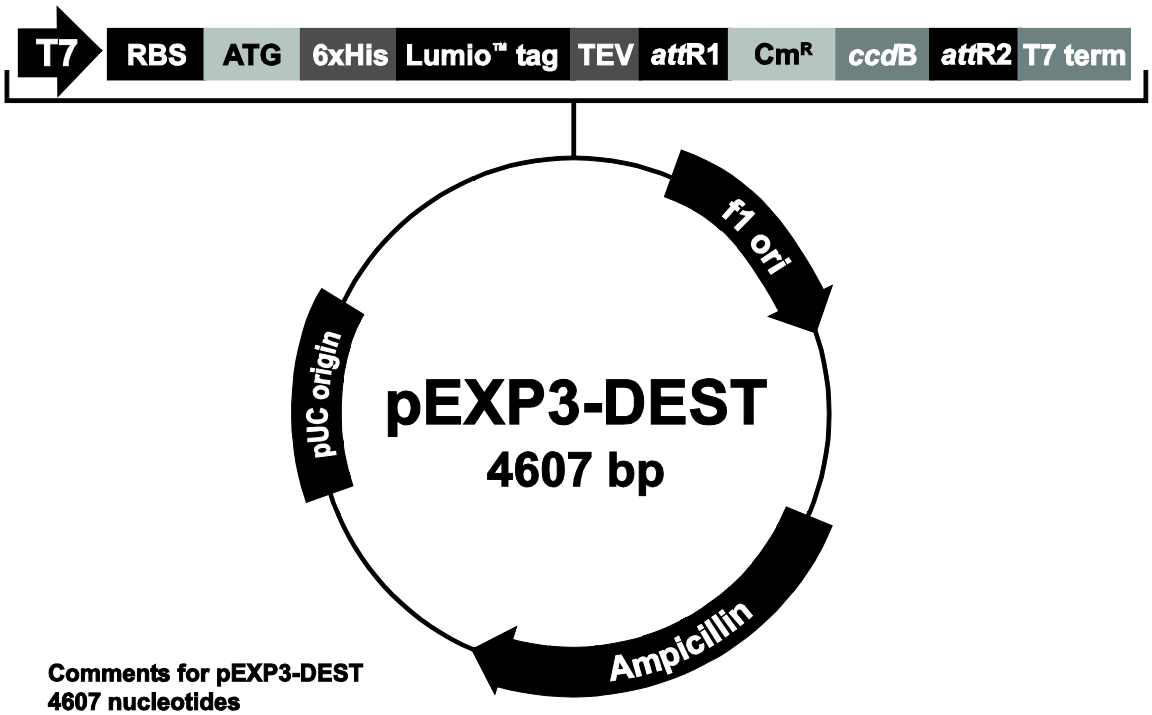
10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.5 with 5 M NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
3. Thaw Zeocin™ on ice and vortex before removing an aliquot.
4. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 μg/ml final concentration.
5. Store plates at +4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

Map and Features of pEXP3-DEST

Map of pEXP3-DEST

The map below shows the elements of pEXP3-DEST. DNA from the entry clone replaces the region between bases 194 and 1877. The complete sequence for this vector available from www.invitrogen.com or by contacting Technical Support (page 44).



Comments for pEXP3-DEST
4607 nucleotides

T7 promoter/priming site: bases 20-39
Ribosome binding site: bases 85-92
Initiation ATG: bases 100-102
Polyhistidine (6xHis) region: bases 103-120
Lumio™ tag: bases 133-150
TEV recognition site: bases 160-180
*att*R1 site: bases 187-311
Chloramphenicol resistance gene (Cm^R): bases 420-1079
*ccd*B gene: bases 1421-1726
*att*R2 site: bases 1767-1891
T7 transcription termination region: bases 1912-2041
T7 reverse priming site: bases 1951-1970
f1 origin: bases 2112-2567
bla promoter: bases 2654-2752
Ampicillin (*bla*) resistance gene: bases 2753-3613
pUC origin: bases 3758-4431

Continued on next page

Map and Features of pEXP3-DEST, continued

Features of pEXP3-DEST

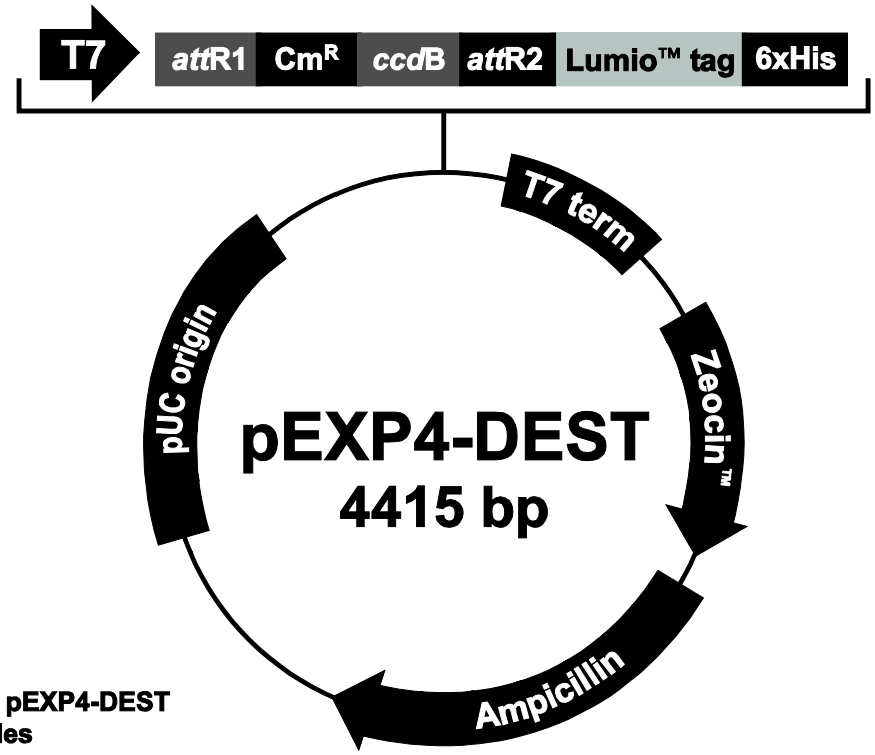
The pEXP3-DEST vector (4607 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
T7 promoter	Permits high-level, inducible expression of your recombinant protein in the Expressway™ Systems or in *E. coli* strains expressing the T7 RNA polymerase.
T7 promoter/priming site	Allows sequencing in the sense orientation.
Ribosome binding site	Optimally spaced from the initiation ATG for efficient translation of PCR product.
Initiation ATG	Allows translation initiation of the recombinant fusion protein.
N-terminal polyhistidine (6xHis) tag	Permits purification of recombinant fusion protein on metal-chelating resin (*e.g.* ProBond™ or Ni-NTA). In addition, it allows detection of recombinant protein with the Anti-HisG Antibodies.
Lumio™ tag (Cys-Cys-Pro-Gly-Cys-Cys)	Allows detection of the fusion protein by the binding of the biarsenical Lumio™ Green Detection Reagent (Adams *et al*, 2002).
TEV recognition site	Allows removal of the N-terminal tag from your recombinant fusion protein using recombinant AcTEV Protease available from Invitrogen (Catalog nos. 12575-015 and -023) (Carrington and Dougherty, 1988; Dougherty *et al.*, 1988).
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Permits negative selection of the plasmid.
T7 transcription termination region	Sequence from bacteriophage T7 that permits efficient transcription termination.
T7 reverse priming site	Permits sequencing in the anti-sense orientation.
f1 origin	Allows rescue of single-stranded DNA.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	Permits high-copy replication and maintenance in *E. coli*.

Map and Features of pEXP4-DEST

Map of pEXP4-DEST

The map below shows the elements of pEXP4-DEST. DNA from the entry clone replaces the region between bases 105 and 1788. The complete sequence for this vector is available from www.invitrogen.com or by contacting Technical Support (page 44).



Comments for pEXP4-DEST
4415 nucleotides

T7 promoter: bases 20-36
T7 promoter/priming site: bases 20-39
attR1 site: bases 98-222
Chloramphenicol resistance gene (Cm^R): bases 331-990
ccdB gene: bases 1332-1637
attR2 site: bases 1678-1802
Lumio™ tag: bases 1831-1848
Polyhistidine (6xHis) region: bases 1864-1881
T7 reverse priming site: bases 1935-1954
T7 transcription termination region: bases 1940-2024
Zeocin™ resistance gene: bases 2168-2542
Ampicillin resistance gene: bases 2563-3423
pUC origin: bases 3568-4241

Continued on next page

Map and Features of pEXP4-DEST, continued

Features of pEXP4-DEST

The pEXP4-DEST vector (4415 bp) contains the following elements. Features have been functionally tested and the vector fully sequenced.

Feature	Benefit
T7 promoter	Allows high-level, inducible expression of your recombinant protein in the Expressway™ Systems or in *E. coli* strains expressing the T7 RNA polymerase.
T7 promoter/priming site	Allows sequencing in the sense orientation.
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Allows negative selection of the plasmid.
Lumio™ tag (Cys-Cys-Pro-Gly-Cys-Cys)	Allows detection of the fusion protein by the binding of the biarsenical Lumio™ Green Detection Reagent (Adams *et al*, 2002).
Polyhistidine (6xHis) tag	Allows purification of recombinant fusion protein on metal-chelating resin (*e.g.* ProBond™ or Ni-NTA). In addition, allows detection of recombinant protein with the Anti-His(C-term) Antibodies.
T7 reverse priming site	Allows sequencing in the anti-sense orientation.
T7 transcription termination region	Sequence from bacteriophage T7 that permits efficient transcription termination.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	Allows high-copy replication and maintenance in *E. coli*.

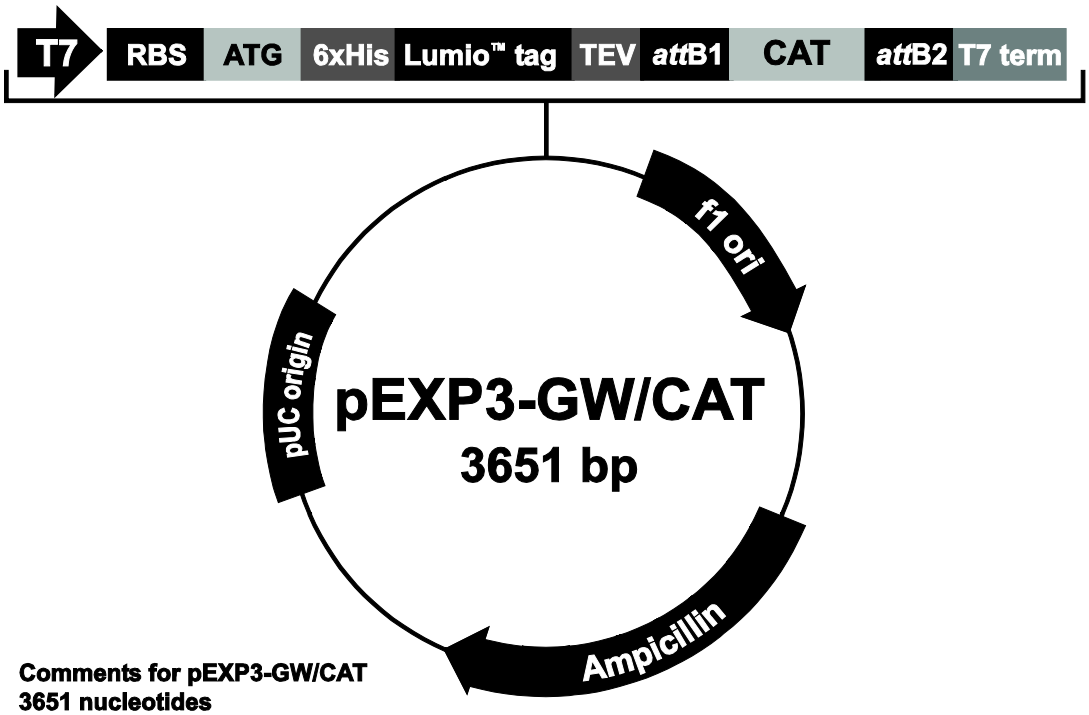
Map of pEXP3-GW/CAT

Description

pEXP3-GW/CAT is a 3651-bp control vector expressing the chloramphenicol acetyltransferase (CAT) protein. The CAT gene is cloned in optimal configuration for expression using the Expressway™ Lumio™ Cell-Free Expression and Detection System. The molecular weight of the CAT fusion protein is approximately 30 kDa.

Map of pEXP3-GW/CAT

The map below shows the elements of pEXP3-GW/CAT. The complete sequence of the vector is available from www.invitrogen.com or by contacting Technical Support (page 44).



Comments for pEXP3-GW/CAT
3651 nucleotides

T7 promoter/priming site: bases 20-39
Ribosome binding site: bases 85-92
Initiation ATG: bases 100-102
Polyhistidine (6xHis) region: bases 103-120
Lumio™ tag: bases 133-150
TEV recognition site: bases 160-180
*att*B1 site: bases 187-211
CAT gene: bases 232-891
*att*B2 site: bases 911-935
T7 transcription termination region: bases 956-1085
T7 reverse priming site: bases 995-1014
f1 origin: bases 1156-1611
bla promoter: bases 1698-1796
Ampicillin (*bla*) resistance gene: bases 1797-2657
pUC origin: bases 2802-3475

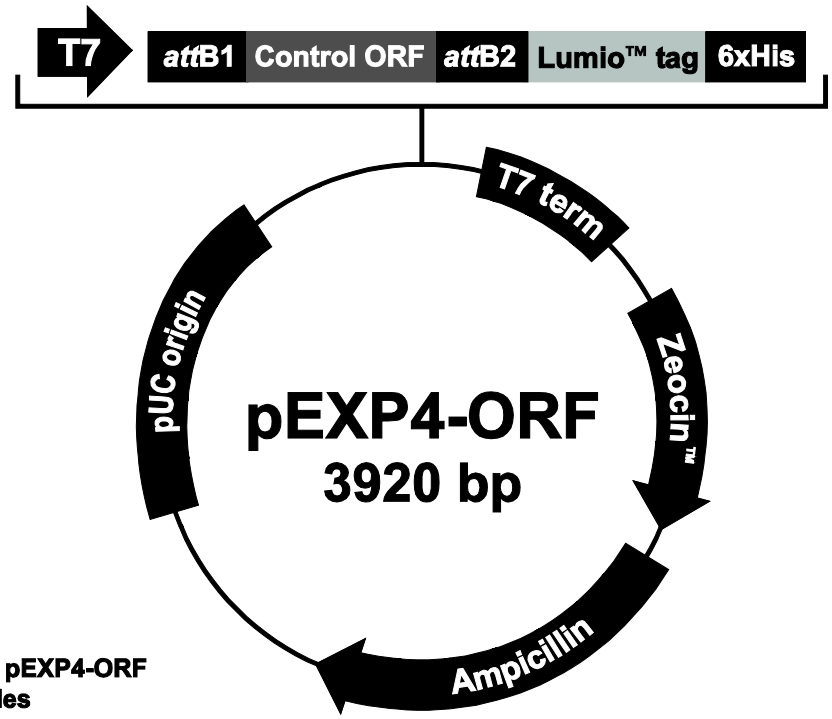
Map of pEXP4-ORF

Description

pEXP4-ORF is a 3920 bp control vector expressing a human open reading frame and was generated using the LR recombination reaction between an Invitrogen Ultimate™ hORF entry clone and pEXP4-DEST. The molecular weight of the native protein is approximately 43 kDa. The molecular weight of the protein fused to the C-terminal tag is approximately 46.5 kDa.

Map of pEXP4-ORF

The map below shows the elements of pEXP4-ORF. The complete sequence of the vector is available from www.invitrogen.com or by contacting Technical Support (page 44).



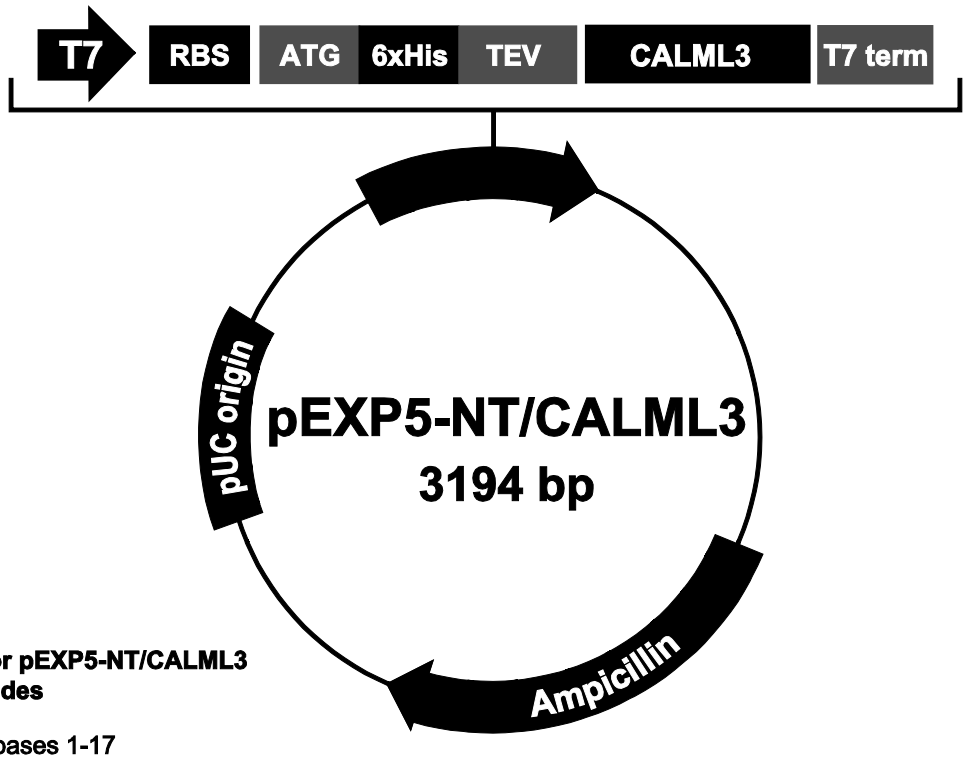
Comments for pEXP4-ORF
3920 nucleotides

T7 promoter: bases 20-36
T7 promoter/priming site: bases 20-39
*att*B1 site: bases 98-122
Control ORF: bases 139-1284
*att*B2 site: bases 1286-1307
Lumio™ tag: bases 1336-1353
Polyhistidine (6xHis) region: bases 1369-1386
T7 reverse priming site: bases 1440-1459
T7 transcription termination region: bases 1445-1529
Zeocin™ resistance gene: bases 1673-2047
Ampicillin resistance gene: bases 2068-2928
pUC origin: bases 3073-3746

Map and Features of pEXP5-NT/CALML3

pEXP5-NT/CALML3 Map

The pEXP5-NT/CALML3 vector (3194 bp) contains a human calmodulin-like 3 gene (CALML3; GenBank accession number NM_005185) that has been TOPO® Cloned into pEXP5-NT/TOPO® in frame with the N-terminal tag. The size of the CALML3 fusion protein is approximately 19.5 kDa. The complete sequence of pEXP5-NT/CALML3 is available for downloading from www.invitrogen.com or by contacting Technical Support (see page 44).



Comments for pEXP5-NT/CALML3
3194 nucleotides

T7 promoter: bases 1-17
T7 forward priming site: bases 1-20
Ribosome binding site (RBS): bases 68-73
Initiation ATG: bases 80-82
Polyhistidine (6xHis) region: bases 92-109
HisG epitope: bases 92-112
TEV recognition site: bases 122-142
CALML3: bases 146-595
T7 reverse priming site: bases 647-666
T7 transcription terminator: bases 608-736
bla promoter: bases 848-946
Ampicillin resistance gene: bases 947-1807
pUC origin: 1952-2625

Technical Support

Obtaining support

For the latest services and support information for all locations, go to www.lifetechnologies.com.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
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- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

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Introduction

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Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase™ from Invitrogen is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

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